

# READING THE LEAVES: A COMPARISON OF LEAF RANK AND AUTOMATED AREOLE MEASUREMENT FOR QUANTIFYING ASPECTS OF LEAF VENATION<sup>1</sup>

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The reticulate venation that is characteristic of a dicot leaf has excited interest from systematists for more than a century, and from physiological and developmental botanists for decades. The tools of digital image acquisition and computer image analysis, however, are only now approaching the sophistication needed to quantify aspects of the venation network found in real leaves quickly, easily, accurately, and reliably enough to produce biologically meaningful data. In this paper, we examine 120 leaves distributed across vascular plants (representing 118 genera and 80 families) using two approaches: a semiquantitative scoring system called “leaf ranking,” devised by the late Leo Hickey, and an automated image-analysis protocol. In the process of comparing these approaches, we review some methodological issues that arise in trying to quantify a vein network, and discuss the strengths and weaknesses of automatic data collection and human pattern recognition. We conclude that subjective leaf rank provides a relatively consistent, semiquantitative measure of areole size among other variables; that modal areole size is generally consistent across large sections of a leaf lamina; and that both approaches—semiquantitative, subjective scoring; and fully quantitative, automated measurement—have appropriate places in the study of leaf venation.

**Key words:** automated image quantification; leaf architecture; leaf rank; leaf venation; repeatability.

“Leaves are all my darker mood.”  
—Robert Frost, “Leaves Compared with Flowers”

The veins in vascular plant leaves distribute water and nutrients (Sack and Holbrook, 2006), transport photosynthate (Giaquinta, 1978), and provide structural support and reinforcement of photosynthetic tissue (Givnish, 1978), under the constraints of existing developmental pathways and phylogenetic history. Leaves are among the most commonly fossilized plant

organs (DiMichele and Wing, 1988; Behrensmeyer et al., 1992), and they often preserve vein networks in some detail. Venation patterns are therefore potentially useful for taxonomic identification, for examination of evolutionary change through time, and for inferring the physiology and habitat of both ancient and modern plants.

There are several independent groups of plant scientists interested in leaf venation: systematists using venation patterns to identify plants and ascertain their phylogenetic relationships; ecologists and ecophysiologicals focusing on the functional role of veins in transport, structural support, and photosynthesis; and developmental biologists examining the pathways and signaling that cause vascular differentiation. This paper is not an attempt to unify these approaches; the classification, quantification, and terminology employed by each of these three groups will probably continue to differ. Here we present an attempt to look at leaf venation from a descriptive but quantitative framework, without taking a position on causality or the importance of the characteristics being quantified. Subsequent study will focus on the ecological and phylogenetic signals that we have observed in the data presented here; in this paper, we focus on methodological issues and try to take no a priori stance on the biological significance or objective reality of the patterns we observe.

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The late Leo J. Hickey does not appear as a co-author of this paper only because we feel that despite his contributions he would not have wanted to be credited as the author of a manuscript that he did not have a chance to edit. He contributed some of the data and many helpful ideas that are reported here. We deeply regret that he did not survive to see the outcome of this investigation, and we would not be comfortable publishing it without emphasizing his contribution. J. Weitz also made significant, practical contributions to the areole measurements discussed here. Part of the work was supported by a Smithsonian Institution postdoctoral fellowship to W.A.G. Thanks also to two anonymous reviewers for their input.

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## PRIOR WORK

The vast majority of prior work on leaf venation has been essentially qualitative (von Ettingshausen, 1861; Hickey, 1971, 1973, 1974, 1977; Hickey and Wolfe, 1975; Doyle and Hickey, 1976; Melville, 1976; Hickey and Doyle, 1977), and the approach taken by plant taxonomists and systematic paleobotanists continues to be primarily descriptive (Ash et al., 1999; Ellis et al., 2009). When appropriate, we will employ the terminology in the most recent version of the *Manual of Leaf Architecture* (Ellis et al., 2009), which provides a brief discussion of the history of the qualitative approach to leaf description. The description of leaves by reference to their venation goes back at least to Linnaeus, who described, for instance, “nervis lateralibus” (Linnaeus, 1753, p. 2) in the net-veined monocot *Curcuma* L. (“lateral veins”; probably actinodromous secondary veins according to Ellis et al. [2009]), and “nervis transversalibus” (Linnaeus, 1753, p. 208) in the dicot *Cerbera* L. (“transverse veins”; which seem to correspond to opposite percurrent tertiary veins in Ellis et al. [2009]). Terminology has changed through time, so what Sargent (1905) describes as “midribs and primary veins” (Sargent, 1905, p. 512) would now be considered primary and secondary veins (Ellis et al., 2009).

Quantifying venation is particularly important for functional or ecophysiological examination (Price et al., 2012; Sack and Scoffoni, 2013). As has been recognized for some time, evidence as to function or adaptation, such as the covariation of phenotype and environment, can be particularly unpersuasive when unsupported by quantitative data (Gould and Lewontin, 1979). Tools for phylogenetic reconstruction also increasingly employ measures of similarity—genetic or morphological—rather than discrete, descriptive characters. For instance, the presence of what is typically called “multiple orders of leaf venation” has long been one of most effective practical diagnostic vegetative characters of angiosperms, and the associated high vein density only observed among angiosperms has recently been implicated in their evolutionary success via a physiological argument (Feild et al., 2004, 2009; Boyce et al., 2009; Brodribb et al., 2010). Recent advances (Katifori and Magnasco, 2012; Mileyko et al., 2012; Price et al., 2013) build on approaches developed for describing river networks, such as the Horton-Strahler method (Horton, 1945; Strahler, 1957). These approaches, however, require a complete description of the network to be employed, and can result in orderings that differ from those obtained using traditional approaches. For example, what would be considered a single first-order vein (primary vein or midrib) under traditional approaches may be composed of several orders using a Horton-Strahler type approach. Moreover, such approaches may be impractical for many situations (e.g., working with leaf fragments or in the field). Thus, it remains nontrivial to define the precise meaning of “multiple orders of leaf venation,” although it is a feature recognized by all botanists. Thus there remain considerable challenges associated with measuring aspects of a vein network accurately, and even greater challenges in characterizing the relationships between measurable variables and their associated functions or phylogenetic significances.

Even defining what a vein consists of can be controversial. It is generally accepted that all veins include some vascular tissue (xylem), but there is more to a vein than just the presence of conducting tissue (Wylie, 1952). Can a single tracheid be considered a vein, or must phloem also be present? Should fibers

and parenchymatous bundle sheath cells be included in the circumscription? In this paper, we take a simple operational view: a vein is a vascular bundle that stains significantly darker than the surrounding ground tissue with standard histological stains like safranin or phloroglucinol. Typically a vein includes tracheids, vessels, associated fibers, and some bundle sheath cells, but this operational definition sometimes includes fibrous bundles that stain dark while lacking vascular tissue or excludes thin vascular strands that cannot be distinguished from surrounding parenchyma except under high magnification. Our definition seems to be approximately accurate in a majority of woody dicot leaves, but it may be more biased in the case of “problematic leaves”—that is, leaves with thick or lignified cuticle or hairs, weak vascularization, extremely large or small size, or atypical lobation or compounding. All of these characteristics make it difficult to obtain reasonably clear images of vein networks. Because these features are not randomly distributed across plants, any collection of specimens or images and the statistics associated with such collections may be biased by nonrandom sampling. In particular, because of the apparent self-similarity of a branching network, aggregate characteristics like vein length will be heavily influenced by the very smallest veins. Capturing images that record vein detail at a cellular level—a scale at which vein geometry clearly influences water transport and photosynthesis—is itself a significant challenge. Full evaluation of these biases and their biological significance is not possible here, but it should be borne in mind both that a quantitative description is only as good as the image from which it is produced and that the description may be biased by choices made in the course of image processing (Price et al., 2014).

In theory, the whole vein system of a leaf can be represented by a mathematical abstraction—a typical reticulate or anastomosing vein network from an angiosperm as a *simple cyclic connected graph*, or the dichotomizing venation like that in most ferns as a *simple acyclic connected graph* or mathematical *tree* (Mileyko et al., 2012). The internodes or segments in such a graph can be named by their connectivity, with the largest vein (midrib) considered primary and higher orders increasing away from the petiole, or alternatively with the finest segments considered primary and connections counted up toward the petiole, as is done by geomorphologists classifying streams (Strahler, 1957). Measurements of the course and diameter of each vein segment (width, thickness, stoutness, and gauge are all synonyms for diameter in this context) should in theory fully characterize a vein network (in either two or three dimensions), although it is not clear that all of the information in such a “complete” characterization is biologically relevant or if such a representation captures everything of biological interest.

In dicot leaves, this sort of “complete” description has not been attempted until recently; for even a small, simple leaf, the time and labor required for full measurement would be prohibitive without automation. This fact was relied on by printers of the early continental currency in colonial North America, who used leaf prints on the reverse sides of bills as an antiforgery device (Newman, 1964). Instead of a complete quantitative description, the most frequently used descriptive terminology for angiosperm leaves is based on what is called *vein order*. This is a venerable but still somewhat vague concept that was developed to distinguish large from small segments in an anastomosing vein system. Up to five orders of venation in certain leaves have been recognized

for some time (von Ettingshausen, 1861). Recent authorities recognize “between four and seven orders” (Ash et al., 1999) of venation in most angiosperm leaves according to the following system: first, all vein widths are measured at their basal junction (i.e., thickest end). The thickest (largest diameter) vein is the main primary vein. The second-largest basal or approximately basal vein is considered primary if its diameter is greater than or equal to 3/4 the size of the main primary vein. If there are two or more primary veins, then other basal or approximately basal veins are considered primary if their diameters are greater than or equal to 1/4 the size of the main primary vein. Secondary veins are structural (branching) elements that do not meet the criteria for recognizing primary or tertiary veins. Tertiaries are the “widest veins that fill the field of the leaf” (Ash et al., 1999, p. 26). They “fill intercostal areas” and “cover the leaf lamina” or “form the vein field mesh or fabric of the leaf” (Ellis et al., 2009, p. 53). For orders higher than tertiary, the same 3/4 rule at the basal junction applies. That is, where a vein branches symmetrically (the smaller branch is greater than or equal to 3/4 the size of the larger branch), no higher order is recognized; where a vein branches asymmetrically (the smaller branch is less than 3/4 the size of the larger), a higher order is recognized. Higher orders are only recognized in anastomosing veins; all veins that branch strictly dichotomously are lumped into the highest order.

While this prescription has often proved to be repeatable by trained observers, it admittedly has a “somewhat arbitrary aspect” (Ellis et al., 2009, p. 47), and some of the authors of this paper would argue that there exists scant empirical evidence for the presence of more than three consistently recognizable vein orders. Three orders is also the number suggested by traditional developmental theory: primary and secondary venation are laid down respectively by apical and marginal meristems while intercalary (plate meristematic) growth accounts for tertiary and any higher orders. In general terms, this three-level hierarchy has been supported by recent developmental work (Poethig, 1984; Nelson and Dengler, 1997; Tsiantis and Langdale, 1998; Piazza et al., 2005).

No classification of veins on a purely functional basis seems to have been attempted (but see Wylie [1938, 1939, 1943, 1946, 1949, 1951, 1952, 1954]). Physiological experiments and models, however, have begun to explore the relationship between vein density (in units of Vein Length per lamina Area, or VLA) and water use efficiency (Feild et al., 2004, 2009; Sack and Holbrook, 2006; Noblin et al., 2008; Boyce et al., 2009). A careful examination of the measurement and significance of vein density is beyond the scope of this paper, but has been begun by Price et al. (2014) and may form the basis of future work. Another variable that could be examined in more detail is total vein length (as opposed to vein segment or internode length), used by Hill (1980) to distinguish secondary veins from “intersecondaries” using the product of their length and width. Defining total vein length, however, is not a trivial problem, and its solution may require more precise definition of vein order than is currently available.

There has also been recent work on the mathematic description of leaf venation in contexts that are not explicitly botanical (Prusinkiewicz and Lindenmayer, 1990; Bohn et al., 2002; Couder et al., 2002; Runions et al., 2005; Dimitrov and Zucker, 2006; Katifori et al., 2010; Katifori and Magnasco, 2012; Mileyko et al., 2012). Of more relevance to our examination,

there have also been prior attempts to combine quantitative leaf description with a biological discussion. For instance, Rolland-Lagan et al. (2009) use automated description of young *Arabidopsis* (DC.) Heynh. leaves to make a limited but interesting developmental argument, and Roth-Nebelsick et al. (2001) provide a general review of the literature on the characterization of leaf venation, along with some preliminary hydraulic modeling results. Price et al. (2012) also provide a useful review of the recent literature along with some conclusions on the scaling properties of leaf vein networks. In this paper, we are concerned not with the choice of formalism used to represent a network mathematically, but with the practical concerns faced in quantifying the venation of real leaves.

## LEAF RANKING

The practice of assigning leaf ranks (Hickey, 1971, 1977; Doyle and Hickey, 1976; Ash et al., 1999) provides a subjective but relatively repeatable method of recognizing levels of organization in leaves: a leaf with few orders of venation and irregular vein courses is considered less organized than a leaf with highly regular veins falling into distinct size classes.

We will not provide a full description here of the practice of leaf ranking, but instead refer the reader to Hickey (1971), Doyle and Hickey (1976), Hickey (1977), and Ash et al. (1999). In brief, leaf ranking involves assigning leaves to one of four arbitrary levels or ranks along a continuum of increasing organization. These four ranks (abbreviated 1r, 2r, 3r, and 4r) can be subdivided into informal, hitherto unpublished, subdivisions (1r1, 1r2, 1r3, [2r0], 2r1, 2r2, 2r3, [3r0], 3r1, 3r2, 3r3, [4r0], 4r1, 4r2, 4r3), where the “nought” ranks in square brackets are transitional between the four main ranks and their use optional. This gives 12 to 15 ordered categories by which leaf organization can be subjectively but semiquantitatively described. In this paper, we treat the full 15-level scale as an ordered factor, or (equivalently) as an integer measure of organization running from 1 to 15. In original conception, the four ranks were associated with organization (regularity of course, regularity of size, regularity of spacing) appearing at the first four orders of veins, so first-rank leaves had regular midribs (but disorganization at higher vein orders); second-rank leaves had regular midribs and secondary veins (but disorganized tertiaries and above); etc. Problems have been observed with this scheme, as when regularity is absent in the primary or secondary veins, but present in the higher-order venation.

Organization as measured by leaf rank, however, does seem to correlate with both phylogeny and ecology. For instance, Hickey (1974) argued that many taxonomic groups show higher organization in their more-derived species, and that the fossil record in general shows a gradient of increasing organization through the Cretaceous. Part of this trend may be due to local environmental and taphonomic bias (facies control), as fossil localities are predominantly from low-land floodplains and will show less variance than modern samples from a broader range of environments. Nonetheless, there does seem to be current consensus that a real evolutionary trend of increasing vascularization is associated with the rise of the angiosperms (Feild et al., 2004, 2009; Boyce et al., 2009).



# Leaf rank in 147 leaves through 8 pairs of eyes

Grey line is mean rank

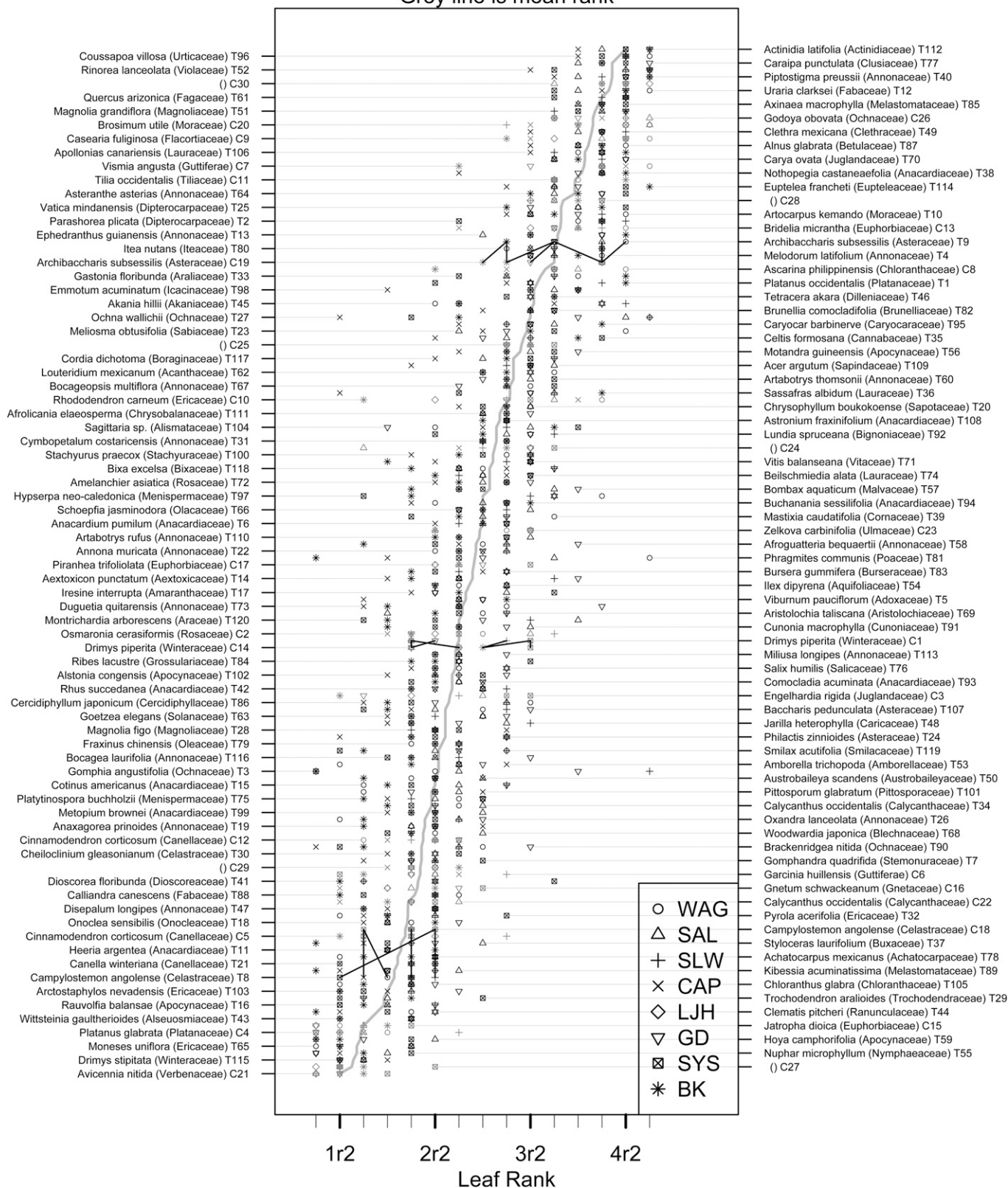


Fig. 1. Raw results of leaf ranking test; leaves ordered by increasing mean rank. Gray plot symbols are control leaves; black are part of the final test set. In three cases, the same image was scored twice and these duplicate scores are connected by solid black lines in this figure.

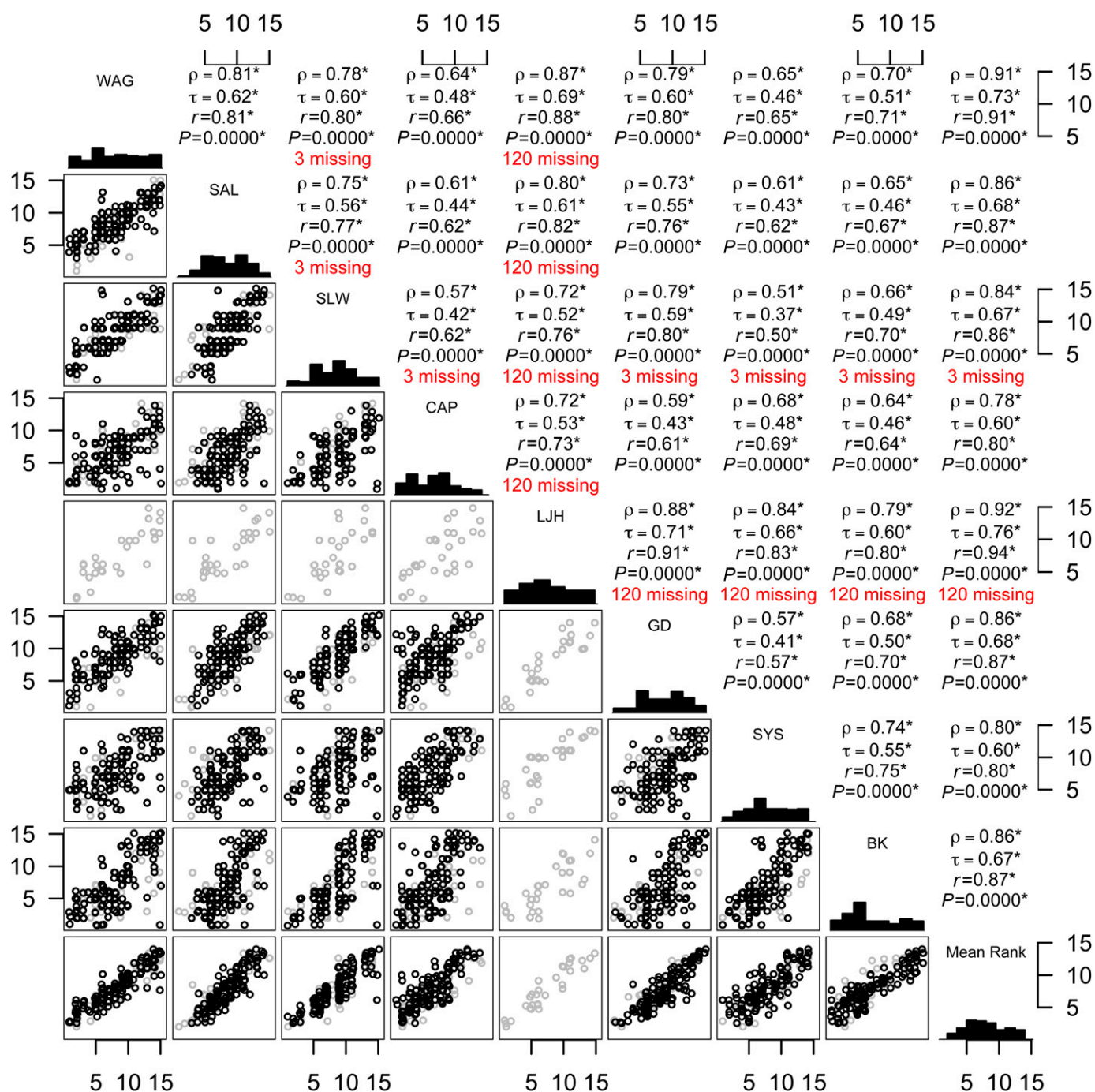


Fig. 2. Comparisons between coders. This is a generalized pairs plot showing, below the diagonal, all the pairwise scatter plots comparing scores of each pair of coders. Each plotted point represents a specimen; the control images are in gray and the final test set of 120 leaves are in black. Above the diagonal are parametric and nonparametric correlation statistics (Spearman's  $\rho$ , Kendall's  $\tau$ , Pearson's product-moment correlation  $r$ ) and the significance of the least-squares regression ( $P$  value). Correlations are significant by all tests and range from about 0.6 to 0.9. Note that the vertical white lines in the scatter plots associated with scores of S.L.W. reflect the omission of the transitional "nought" ranks. See Emerson et al. (2013) for further discussion of the generalized pairs plot. L.J.H. only completed the control sets.

The original intent of leaf rank was primarily to describe the phylogenetic and ecological distribution of leaf organization in angiosperm leaves. The general conclusions reached were that:

"families tend to show one predominating level of organization. Families considered primitive from other evidence

cluster about the lower ranks with the intermediate and advanced families ranked higher, respectively ... most families fall within the third rank. Similar, though smaller, differences in rank occur between the primitive and advanced genera of the better known families (Theaceae).

The fossil record of the angiosperms also shows a gradual increase in the rank of leaf assemblages from the first to the third ranks during Early Cretaceous time.... Finally, leaves of plants native to arid, arctic, or alpine environments frequently show a regression below the organizational level characteristic of the family.” (Hickey, 1977, p. 159)

Leaf ranking has not been very broadly applied, perhaps because it is a coarse measure, reliant heavily on the learned recognition of Gestalt categories. Therefore, it was only considered possible to learn how to apply the system by calibrating one’s own perceptions of organization against a database of leaves with established ranks, which has not until now been available in published form. In this paper, we provide rank scores for 120 leaves along with some data on the expected variation among different coders. Little et al. (2014) and subsequent papers will focus on the systematic and ecological distribution of leaf rank; here we are concerned with establishing its repeatability, expected variation, and how it corresponds to automatic measurements. In the process, we also hope to demystify leaf ranking, which has been considered a sort of black magic, as our results suggest that extensive training is not necessary for reliable application of the method.

## AUTOMATED IMAGE ANALYSIS

Relatively recent advances in computer modeling and fractal geometry have stimulated interest in synthesizing leaf-like networks relying only on simple physical or mathematical models (e.g., Prusinkiewicz and Lindenmayer, 1990; Bohn et al., 2002; Couder et al., 2002; Runions et al., 2005; Dimitrov and Zucker, 2006). From a botanical perspective, the problem with such approaches is that they focus on the type of generality that may be physically or mathematically interesting but tends to demonstrate biological truisms. To the best of our knowledge, no study to date has provided a body of measurements of enough leaves to evaluate the ecological or phylogenetic signal in different measurements, although the study by Wilf et al. (2012) represents a large-scale attempt currently under way to use automated image analysis to identify leaves taxonomically; and the database discussed by Das et al. (2014) could be so examined.

Using photographs of modern cleared leaves and an automated image analysis protocol, we measured a number of attributes of the vein network of 120 leaves, representing most major angiosperm groups and a few ferns. The measurements related to the areoles—the largest regions of leaf lamina

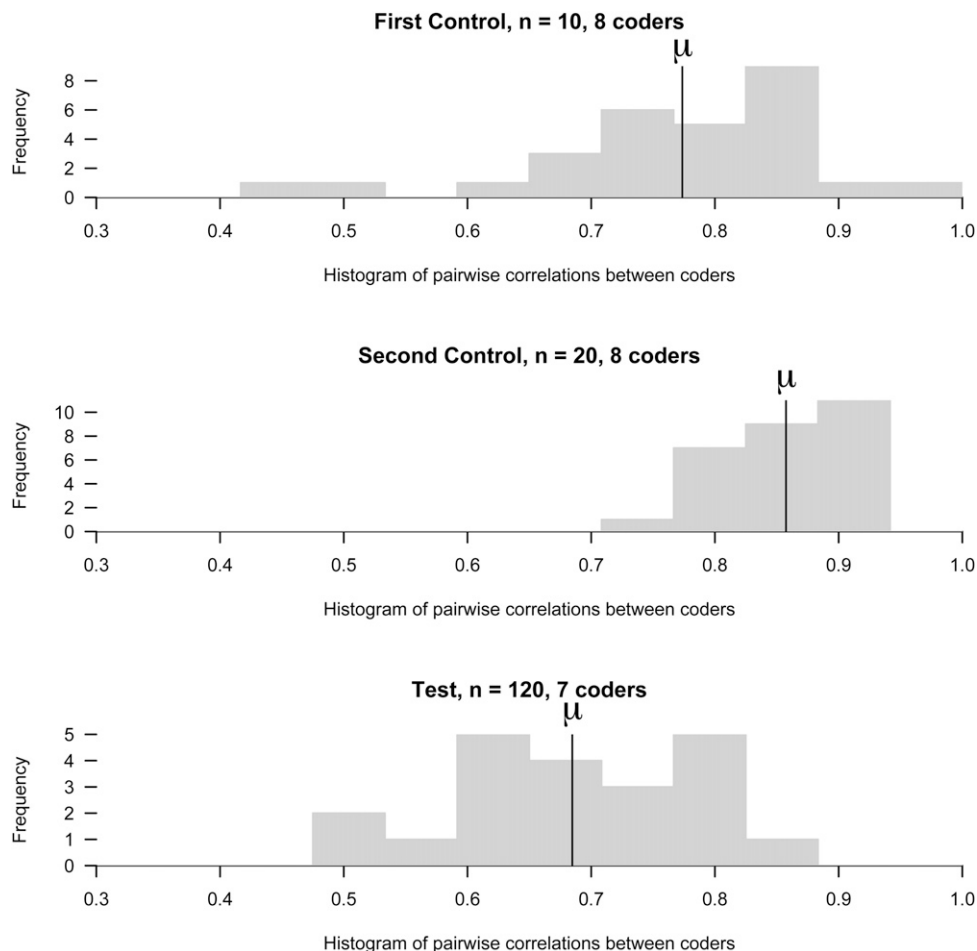


Fig. 3. Histograms of pairwise correlations between coders, broken down in the three coding phases: control 1 (10 leaves, 8 coders), control 2 (20 leaves, 8 coders), and test set (120 leaves, 7 coders). In each case, the mean correlation is indicated. There is significant variation but no strong evidence for systematically increasing correlations through the three phases of coding.



(ground parenchyma) completely surrounded by veins. While such measurements cannot be directly converted to vein diameters and lengths, they reflect an identical underlying geometry in thresholded (binary) images, and therefore should show similar biological signals. As demonstrated below, they are consistent across a leaf lamina and seem to correspond well to intuitive and descriptive notions of vein organization, like areole size and shape.

## METHODS AND RESULTS

**Leaf ranking**—Our set of 120 species was chosen from the cleared leaves available in the National Cleared Leaf Collection (NCLC) at the Smithsonian Institution. Specimens were chosen to represent most major clades among the vascular plants, and were otherwise selected by grab sampling among the available leaves in sufficiently good condition to photograph. In addition to the “test” set of 120 leaves (T1–T120), we also collected two smaller training or “control” sets—the first of 10 leaves (C1–C10) and the second of 20 leaves (C11–C30)—to evaluate the learning process that would be faced when employing a new technique. After scoring each of the two small control sets, the coders compared results and discussed any substantial disagreements about the coding of each leaf before moving on to the final set of 120 species. The coders ranged in experience from no prior experience with examination of leaf venation (C.A.P., B.K.) to over a decade of experience with the specific techniques of leaf ranking (S.L.W., L.J.H.). All scoring was semiblinded: one author (W.A.G.) randomized and renumbered all the leaf images before redistributing them for coding. Three leaves were seen twice by each coder to get a direct measure of repeatability, giving a total of 147 distinct leaves and 150 distinct sets of rank scores. After the first control set, a second, detailed image of the higher-order venation was provided, as well as a photograph of the whole leaf. In general, we seldom differed about the relative ranking of two leaves, but often interpreted the ranking rules slightly differently. This was especially true in the cases of atypical leaves: those with palmate primary veins, very many or very few secondary veins, or the presence of a feature like an intramarginal vein.

Figure 1 shows the raw results of coding with the leaves reordered from bottom to top in order of increasing mean rank. For the three leaves scored twice (unknown to most of the coders), the two occurrences are connected by solid lines, and the first two control sets (C1–C10 and C11–C30) are shown in gray. Although we initially expected there to be more variation among the control leaves and more consistency between coders with the final test set (a “learning” effect), we found no dramatic difference between the consistency with which the control and test sets were coded.

Note the relatively good rank-order consistency of all the scores, slightly higher consistency within the scores from an individual coder, and the relatively large range of variation among coders for some leaves. In general, scores for a single leaf tended to range over a full rank (four subranks), and the only effect of extensive prior experience seemed to be a weak propensity to avoid very high and low ranks. In particular, T81 (*Phragmites communis* Trin.), a parallel-veined monocot, and T3 (*Gomphia angustifolia* Vahl) showed the full range of possible variation. Two ferns (*Onoclea* L. and *Woodwardia* Sm.) and a leaf of *Gnetum* L. were also included, all showing relatively low ranks, although leaf ranking was not originally intended to be applied outside the dicots. Figure 1 also shows, as had been observed anecdotally in the past, that there were small systematic biases introduced by certain coders.

Despite the substantial differences among coders in experience with leaf venation in general and with leaf ranking in particular, the pairwise correlations between coders ranged from 0.6 to 0.9 and did not seem strongly correlated with experience—that is, the more experienced coders did not systematically agree more with each other than the inexperienced coders. This is apparent from Fig. 2, where the control leaves are again shown as the gray points; the test set of 120 in black. All parametric and nonparametric tests for correlation between coders were positive and highly significant.

Also, somewhat surprisingly, there did not seem to be significant overall improvement in consistency over the course of the control and test sets despite the fact that some obvious errors by certain coders were made in the control sets. Figure 3 shows histograms of the pairwise correlations between coders, which vary significantly but do not consistently improve over the course of the experiment.

To evaluate the expected magnitude of random variation introduced, we also resampled 30 leaves from the complete control-plus-test set (150 leaves) 200 times and found a similar random spread. Figure 4 shows the actual distributions of correlations between coders for test leaves (solid line) and control leaves (dotted line) superimposed on the bootstrapped replicates. This shows that if there is any learning effect, it is too small to be identified in this experiment, given the random errors observed.

We conclude from this examination that leaf rank is an imperfect and subjective measurement, but it is largely rank-order consistent across different coders, who tend to show correlations of about 0.7 with each other. Thus, leaf rank is a decent semiquantitative estimator of leaf organization. It is also very quick—the final test set took most coders only a matter of a few hours to complete. Because leaf rank is determined subjectively, it remains difficult to be sure precisely what measurable characteristics of the vein network leaf rank describes.

**Automated image analysis**—The same group of 120 leaves was photographed for automated image analysis using a macrophotographic setup including a macro lens, bellows, and digital camera back on a copy stand. The (true) image size was set to approximately  $1.2 \times 7$  cm with a  $2.4 \times 1.6$ -cm image sensor with  $3008 \times 2000$  pixels (about  $4\times$  optical magnification), giving a true pixel dimension of about  $4.4 \mu\text{m}$  or approximately  $50,000$  pixels/mm<sup>2</sup>. By experiment, we found that this procedure gave clearer, flatter images than the use of a flat-bed scanner or a photographic stereomicroscope.

Where it was possible, two images were obtained for each leaf: one in the center of the lamina, intersected by the midrib, and the other wherever on the leaf lamina the primary and secondary network of major veins could best be avoided. These two images (labeled *a* and *b*) should, therefore, respectively maximize and minimize the influence of the major veins on the intercostal venation. The off-midrib image (*b*) was also used as the detailed image of each leaf in the rank test described above.

Each image was converted to a binary, black-and-white mask (black vein on white background) using an adaptive thresholding algorithm, automatically cleaned, and each connected white region measured using a MATLAB script (The MathWorks Inc., Natick, Massachusetts, USA) with the same underlying functionality as the software described by Price et al. (2011). The details of this processing are provided as a MATLAB script (matlab\_code.txt; available from

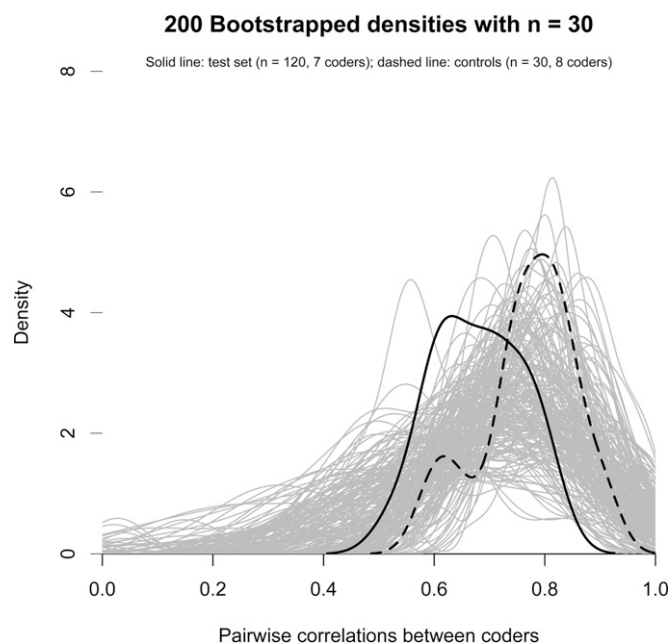


Fig. 4. Distributions of correlations between coders, superimposed on 200 bootstrapped distributions of size  $n = 30$ . The solid line represents the final test set (120 leaves, 7 coders); the dashed line represents controls (30 leaves, 8 coders).

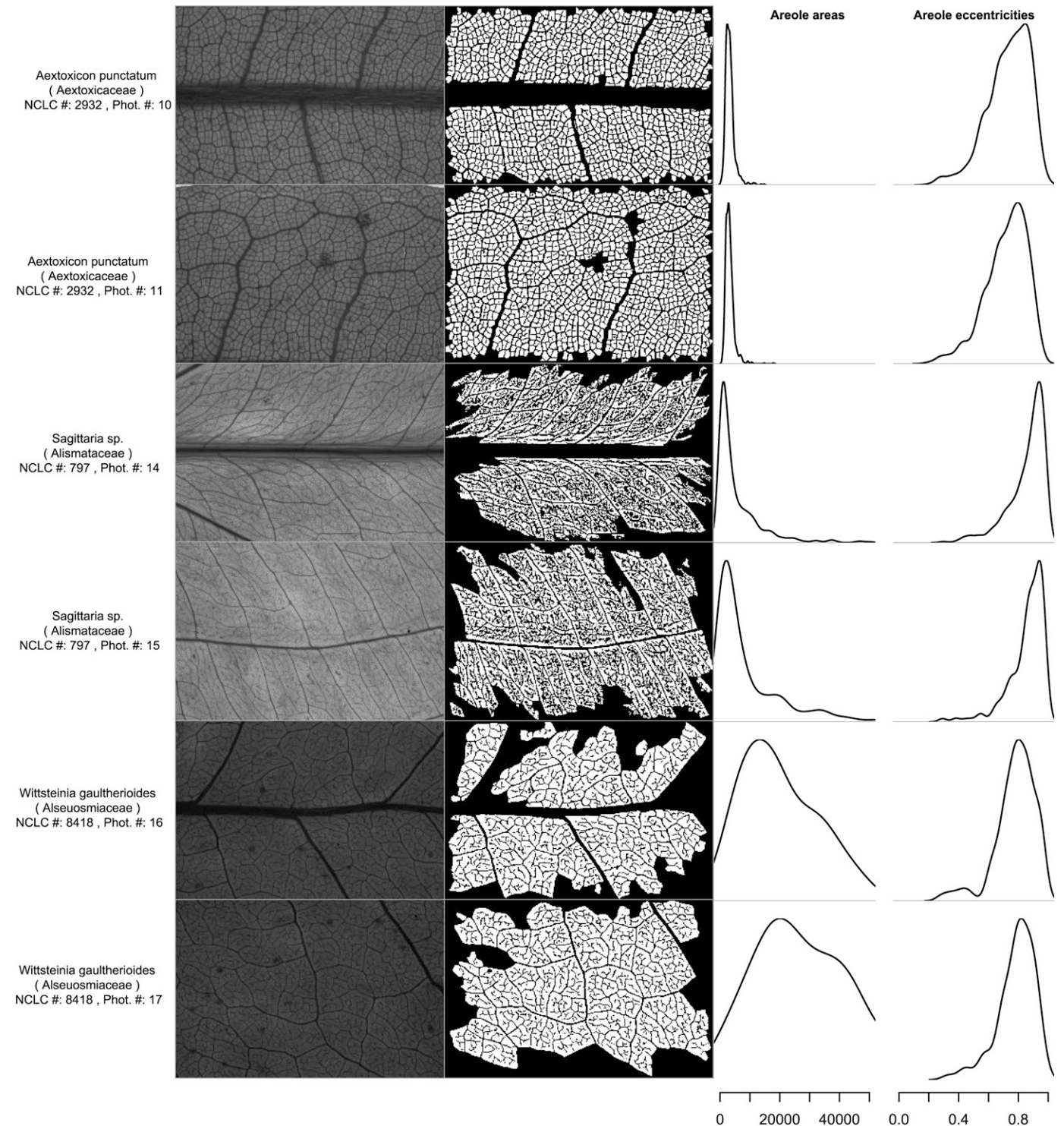


Fig. 5. Images with distributions of areole measurements. For six images of three leaf specimens (on midrib and off midrib), the figure gives the distributions of areole areas (in pixels) and areole eccentricities (dimensionless number between 0 and 1). Y-axes vary and are not meaningful because of the different number of areoles in each image; therefore, they are omitted from this figure. Note the small, narrow range of areole sizes and low eccentricity of areoles in the first two images; the similar-sized but more eccentric areoles in the middle pair; and the larger, more variable areoles in the bottom two images.

the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.8h022>; Green et al., 2014). The variables measured included:

Area: number of pixels in each region

Convex Area: number of pixels in the convex hull of a region

Eccentricity: eccentricity of the ellipse that has the same second-moments as the region

Solidity: proportion of the pixels in the convex hull that are also in the region. Computed as Area/Convex Area.



Perimeter: distance around the boundary of each region  
Equivalent Diameter: diameter of a circle with the same area as the region  
Major Axis Length: length (in pixels) of the major axis of the ellipse that has the same normalized second central moments as the region  
Minor Axis Length: length (in pixels) of the minor axis of the ellipse that has the same normalized second central moments as the region

We measured a total of 208,253 regions (areoles) in 230 images, of which 206 images seemed “good,” i.e., the masks seemed to reflect the general pattern of veins and areoles observed in the original leaves accurately. This subjective assessment of whether a binary mask accurately reflects a vein network is not easily avoidable. As with many biological measurements, there is no gold standard for what constitutes accurate measurement of a feature of the real world; so our own human perception has to serve as ground truth (Martin et al., 2004).

Figure 5 shows six images of three leaves. The first column provides the grayscale image, the second column the binary mask, the third a distribution of the areole sizes, and the fourth a distribution of the areole eccentricities; because the distribution shapes but not the absolute densities are relevant, the y-axis labels and ticks are omitted for clarity.

A complete representation of the raw areole area measurements is shown in Fig. 6. Each measured areole is shown as a gray dot, the superimposed black lines give the interquartile ranges of each image, and the monotonically increasing line connects the modes of the areole area distributions of each leaf. Several features of interest can be observed from this representation: the pattern of right-skewed distributions of areole sizes with a mode on the lower end of the interquartile range is quite general; the mode, therefore, seems to be the best estimate of the central tendency of areole size in each image. The cropping at the bottom of the graph is due to the image cleaning protocol, which removes regions under a given size (in this case, 300 pixels), but notice that this cropping does not affect the modes.

Because most of the raw image measurements are paired (two images per leaf), a useful check is provided by comparing the summary statistics of the

on-midrib (a) images directly with the off-midrib (b) summaries. Figure 7 shows this comparison, with each point representing a leaf labeled with its NCLC catalog number. Note the close correspondence between the images; whether or not potentially problematic images are excluded, the least squares fitted line does not differ significantly ( $P = 0.39$ ) from the  $y = x$  diagonal.

## DISCUSSION

Figure 8 provides a direct comparison between the rank test results shown in Fig. 1 and the areole measurements shown in Fig. 6. This reveals a statistically significant but noisy relationship. As expected, leaf rank provides an approximate method for estimating modal areole area, explaining about 14% of the variance in the areole size measurements (using the squared correlation as a metric). Correlation tests using nonparametric measures of correlation are also significant:  $P < 0.001$  for both Kendall's  $\tau$  and Spearman's  $\rho$ .

Comparing mean rank with the other measured quantities substantiates the idea that rank is not just a proxy for areole size. Of the six measured areole characteristics shown in Fig. 9, leaf rank actually correlates best with areole standard deviation ( $r = 0.59$ ). Superficially, this suggests that our perceived value of leaf rank corresponds best to low variability in areole size. This is a plausible conclusion, but the data may not support it conclusively. Because there are significant correlations between measures of center (mean, median, mode) and

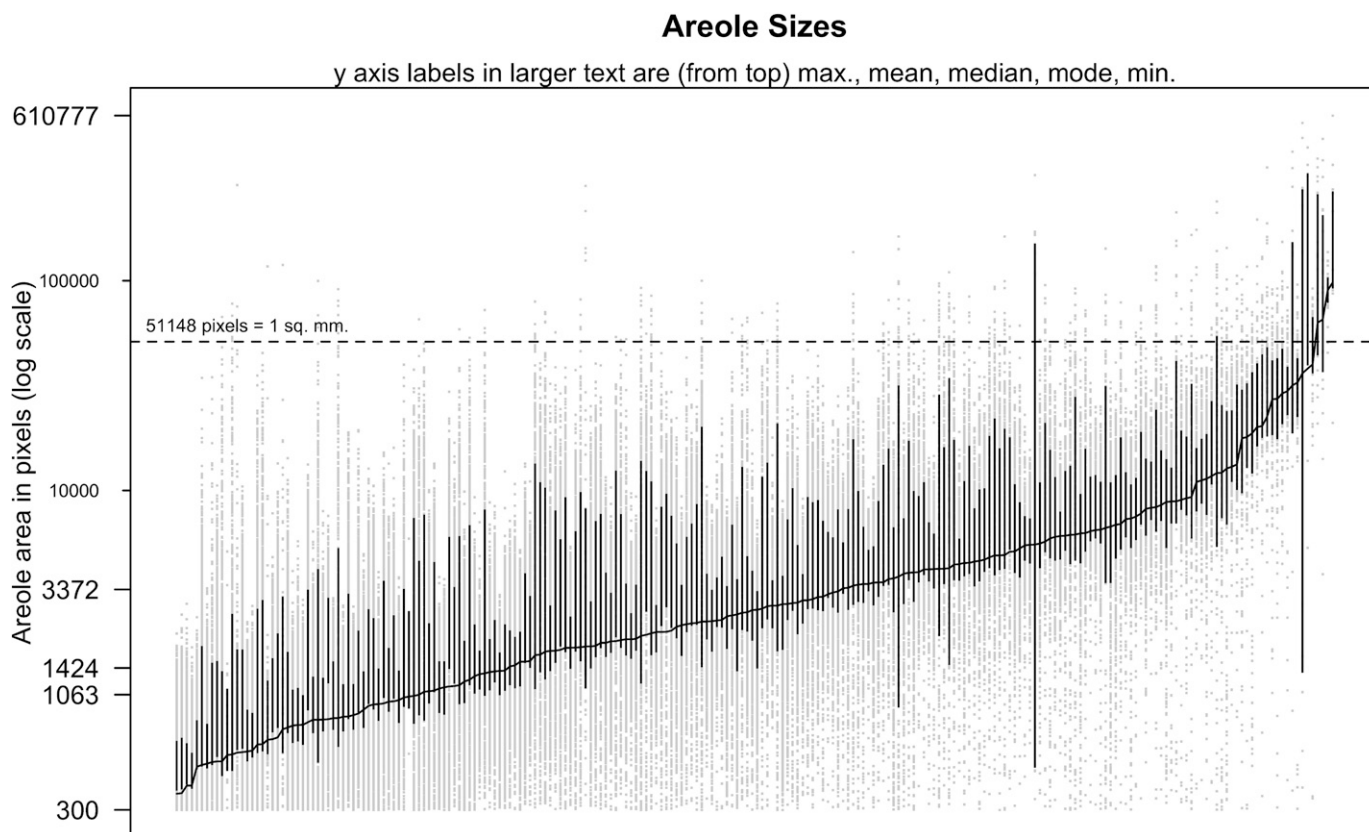


Fig. 6. Raw areole measurements of 230 images representing 120 leaves. Gray dots correspond to raw areole size measurements; black vertical lines are the interquartile range for each image; the monotonically increasing line connects the modal areas for each image. Images are arranged from left to right in order of increasing modal areole size. Note cropping at the bottom caused by the image cleaning protocol and the right skew of the distributions (modes near the bottom of the interquartile ranges).

## Comparison of image pairs

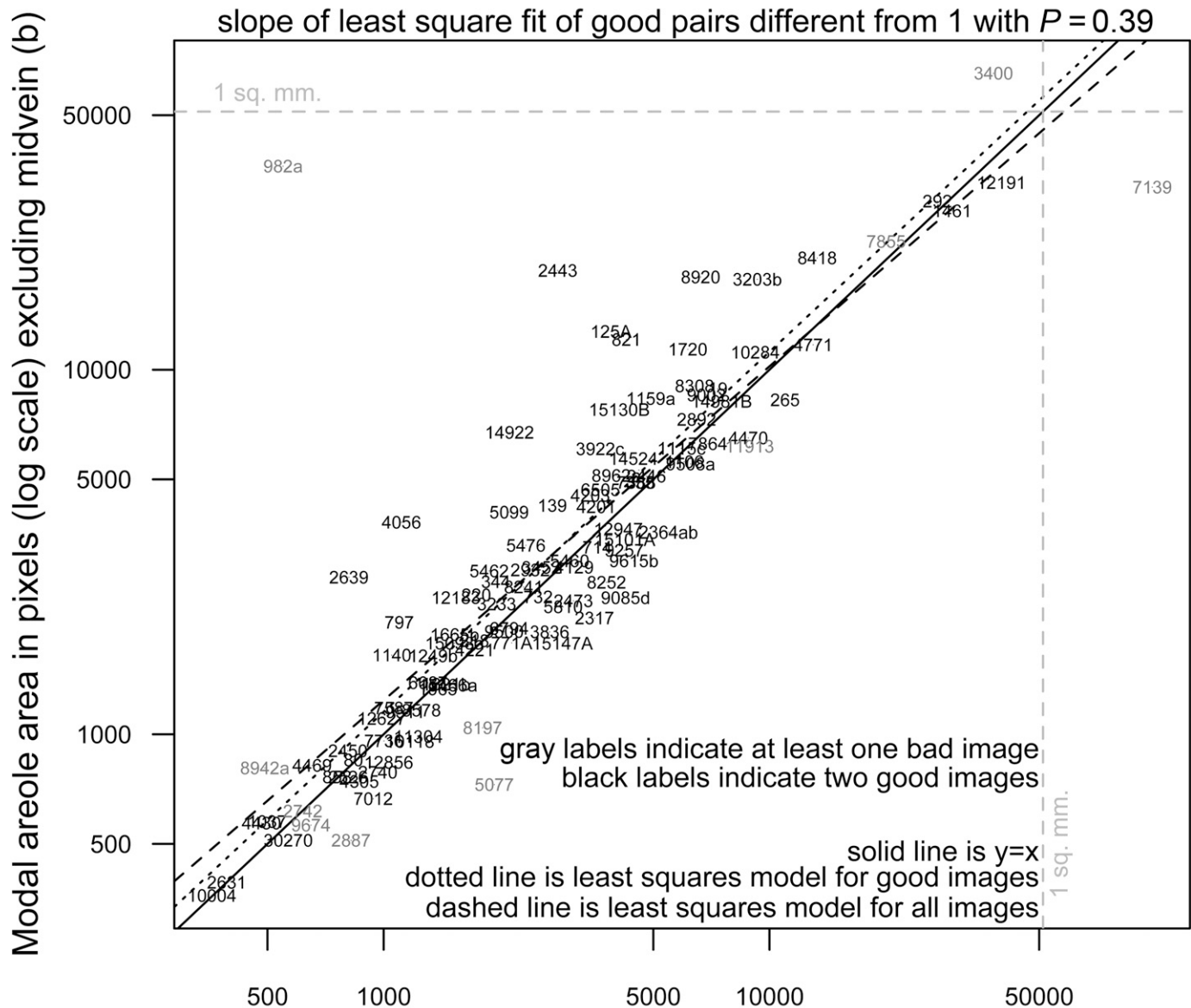


Fig. 7. Comparison of modal areole areas of on-midrib (a) and off-midrib (b) images. Each point represents a leaf specimen and is labeled with its catalog number; points with gray labels have one or more images independently judged poor; black labels identify leaves with two good images; see text for discussion of “good” images. The dashed line is the least squares line fitted to all the data; the dotted line is the least squares line fitted to the subset of good images. Neither differs statistically from the  $y = x$  diagonal (solid line).

measures of spread (standard deviation, interquartile range) throughout our areole measurement data, further investigation is needed before we say precisely what leaf rank is measuring. In any case, attempting to assign a quantitatively precise definition to leaf rank may be missing its utility.

Leaf rank is unlikely to be used extensively as a direct proxy for better-defined measurements like areole area. Instead, like ordinal scales used in medicine (e.g., cancer staging), the method of leaf ranking retains its utility for practical reasons:

data are quickly and easily obtained, even on leaves or leaf images of dubious quality. Its main applications may remain in the study of fossil leaves, where poorly resolved vein networks are often all that is preserved, but it could also prove useful in field botany, where vegetative characters for quick taxonomic identification are important. The system as presented here might well benefit from a simplification and redefinition of ranks: the relatively high average variation among coders—approximately a full rank (four subranks)—suggests

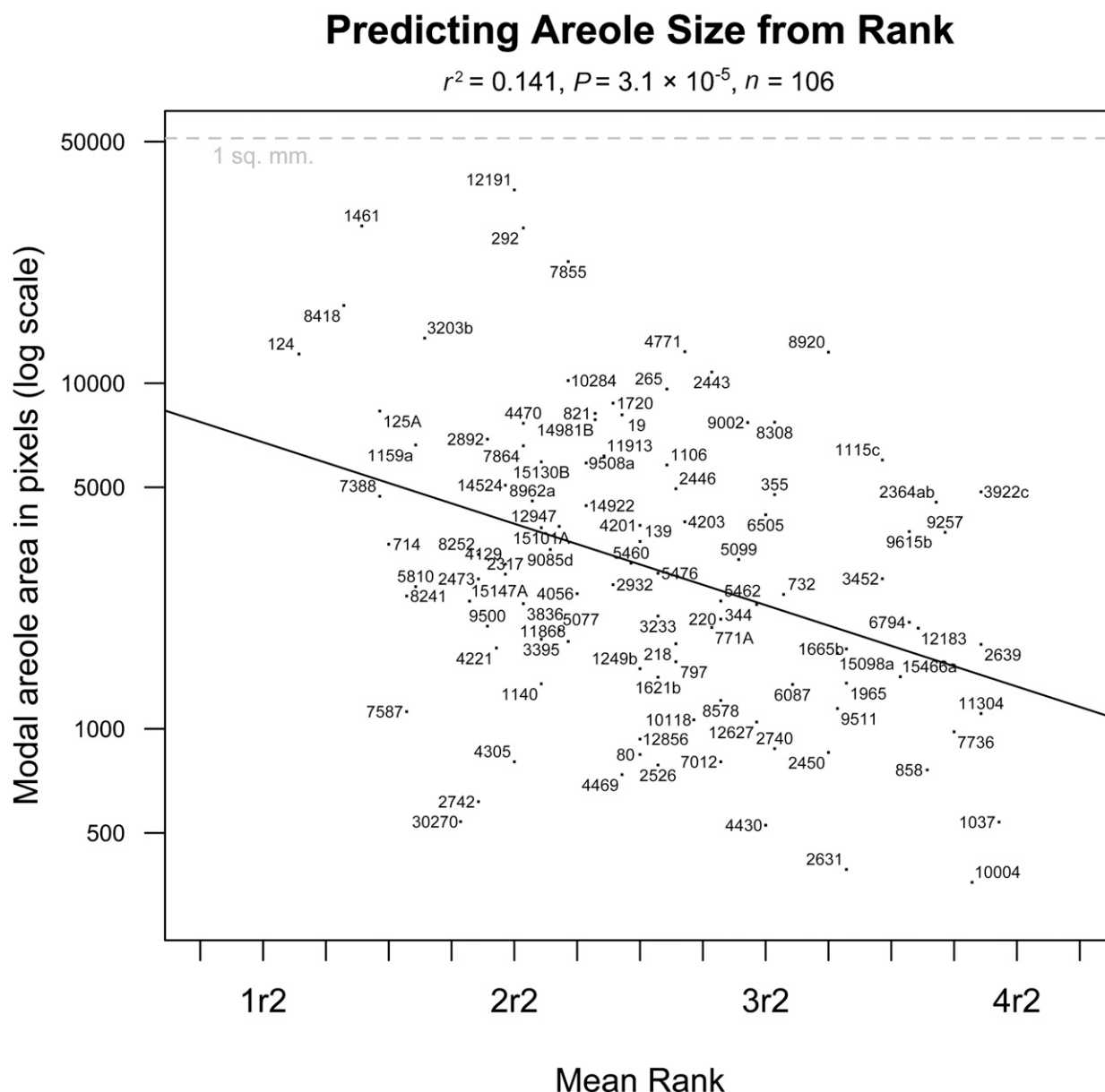


Fig. 8. Comparison of leaf rank with modal areole area. Each point represents a leaf specimen, labeled by its catalog number. The least squares regression line shows a highly significant inverse relationship between leaf rank and areole size explaining about 14% of the observed variance in modal areole area in the 106 of 120 leaves for which two measurements could be made.

that a leaf rank scale with 15 levels (as used here) provides more precision than the accuracy of the coding method can support. The original four ranks implicitly corresponded to “organization” present in the first four vein orders. Because objective recognition of vein orders above third order is doubtful and data presented here suggest that a scale with a total of five to seven levels might be sufficient, an improved leaf ranking system might rely only on separation of primary, secondary, and intercostal venation and be limited to six ranks.

To advance the study of the functional, physiological, or developmental biology of leaf venation, leaf rank is unlikely ever to offer sufficient precision, so automated collection of true measurements is likely to become increasingly dominant.

The barriers to progress in this direction are more practical than theoretical: the technology (both hardware and software) is available to develop the methods described in this paper and apply them to 120,000 leaves instead of 120, with much improved image quality and more effective analytic software. Unfortunately, it remains difficult to scale up in an academic setting. Therefore, studies of leaf venation may continue to lag equivalent governmental or commercial methods by decades merely because the hardware needed to improve imaging and image workflow is expensive and because image analysis software tends to be either compiled and proprietary or composed of heterogeneous code snippets that are poorly documented or difficult to integrate with each other. Our own experience making the measurements described



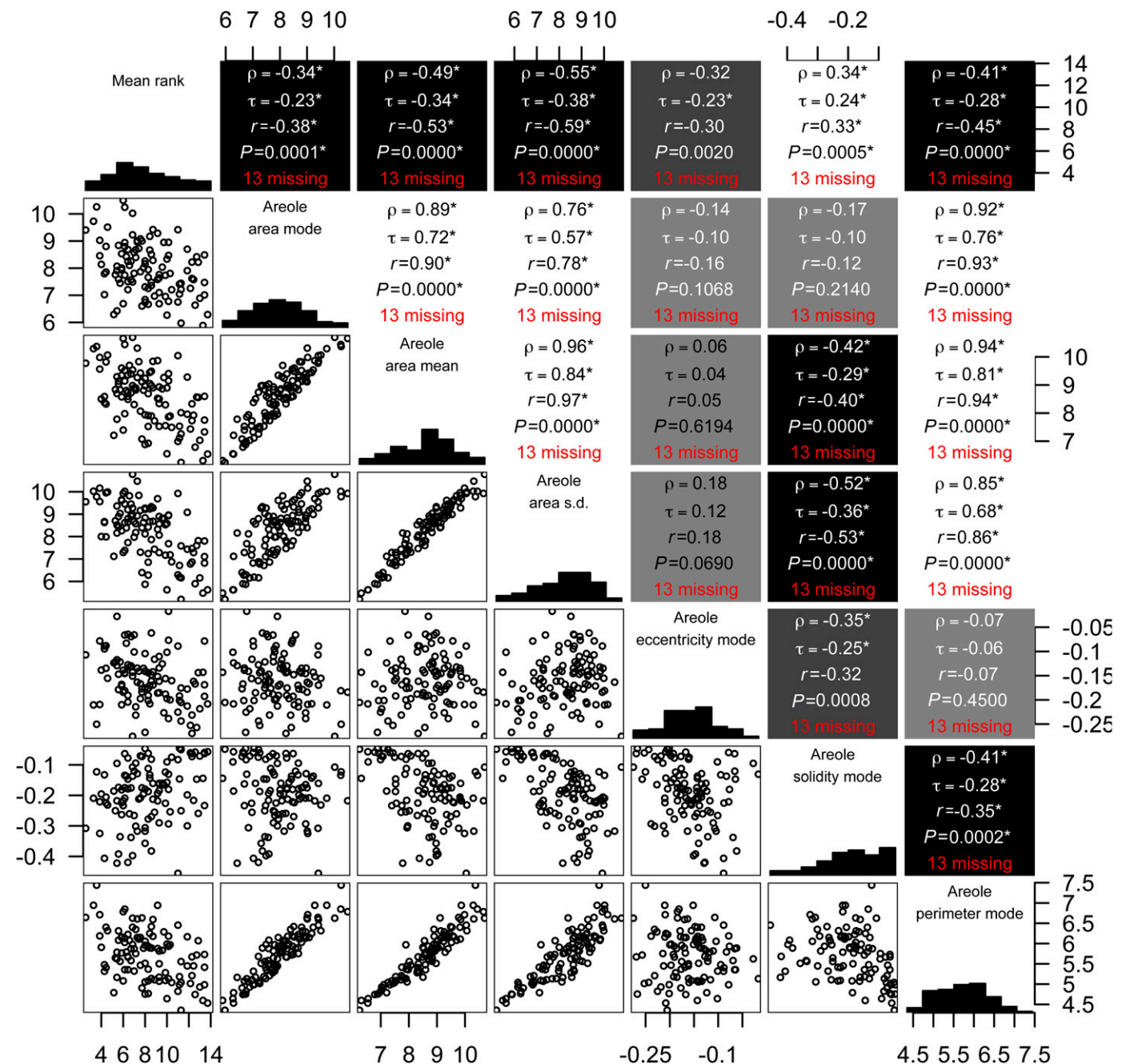


Fig. 9. Generalized pairs plot comparing leaf rank with several other measured areole characteristics. The top left scatter plot is the same plot as Fig. 8. Each point represents a leaf specimen and the other scatter plots below the diagonal show covariation among some of the other measured quantities. Note in particular that the correlation between mean leaf rank and areole standard deviation is substantially higher than the correlation between mean rank and areole size.

here provides a good example of the difficulties that can be encountered. We relied on the underlying image analysis code in the commercial package MATLAB and, therefore, some of our analyses can only be documented by reference to compiled code. The way forward that we would advocate follows the open-source model: public repositories of images and commented source code, documented in sufficient detail for comparatively small contributions to build on each other. Attempts have been made to provide this sort of facility (e.g., <http://clearedleavesdb.org/>, [\[plant-image-analysis.org/\]\(http://plant-image-analysis.org/\)\), but many more publications in this arena describe new software than provide source code for it. In an attempt to provide an example of the procedure that we advocate, we have included in the supplemental archive associated with this paper \(available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.8h022>; Green et al., 2014\) all the images and code used. Because even the simplest data table would run to several pages when printed out, we have not included paper data appendices, but instead refer readers to the supplemental archive, which](http://www.</a></p>
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includes far more information than can be discussed in the text of the paper.

Finally, it should be re-emphasized that automation does not provide objectivity: throughout the process of automatic analysis, choices are made and parameters are set that can bias the measurements in ways that may be unpredictable without extensive sensitivity analysis (e.g., Price et al., 2014). Automation provides data of a sort that would be otherwise unobtainable, but its application also carries the need for thorough documentation of elaborate, complex procedures.

## CONCLUSIONS

From this examination, we conclude that leaf rank can be quickly and repeatably scored by different people without significant training or experience, with a consistency (correlation between pairs of coders) of about 0.7.

It also appears that leaf rank is inversely correlated to areole size and related to other measurable attributes of leaf areoles. Specifically, leaf rank seems to explain about 14% of the variance when used as a predictor of modal areole area in a set of 120 angiosperm leaves. It may be even more closely associated with measures of within-leaf areole variability.

Areole size is generally consistent across the leaf lamina and can be reliably measured on an area as small as 1 cm<sup>2</sup>, provided images of sufficient quality are available. The barriers to quantitative description of leaf venation tend to be practical: the need for images that can be accurately thresholded and the difficulty of completely automating the process of image cleaning and analysis.

Thus, leaf rank remains a useful semi-quantitative proxy for leaf organization when fully quantitative measurements cannot be made, as in the case of most fossilized material. Automated measurements, which will become easier with every advance in digital image acquisition and analysis, provide hitherto unobtainable data with which to investigate functional, physiological, or developmental attributes of modern leaves. It is essential for the progress of such studies to build up public databases of identified, high-quality images and commented source code, documenting analytic choices in full detail.

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